REMARKS

Claims 1, 2, 4-9, and 11-18 are pending after entry of this paper. Claims 1-4, 9-15, and 18 have been rejected. Claims 5-8, 16 and 17 have been withdrawn and claims 3 and 10 have been previously cancelled without prejudice. Applicants reserve the right to pursue withdrawn and cancelled claims in a divisional or continuing application.

Claim 1 has been amended to replace the transitional open-ended term "comprising" with a closed ended transitional term "consisting."

No new matter has been introduced by this response. Reconsideration and withdrawal of the pending rejections are respectfully requested.

Response to Rejections under 35 U.S.C. §103

Claims 1, 2, 9, 11, 12 and 18 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Hsih et al. (*J Food Prot.* 2001 Nov; 64(11):1744-50) in view of Kearney et al. (U.S. 5,589,335), and further in view of Brasher et al. (*Curr Microbiol.* 1998 Aug; 37(2):101-7). Applicants respectfully disagree with the arguments set forth in the Office Action with respect to the cited references and the attained conclusion.

As an initial matter, in order to expedite prosecution and without disclaimer of, or prejudice to, the subject matter recited therein, applicants have amended the presently pending claim 1 to replace the transitional open-ended term "comprising" with a closed ended transitional term "consisting of." Thus, the arguments put forth by the Patent Office that the instant claims allow for inclusion of immunomagnetic separation used by Hsih (Office Action; pg. 6) is now moot.

In response to applicants previous arguments, the Patent Office takes a position that it would have been obvious to a person of ordinary skill in the art to add known lysis agents for known bacteria to the methods of Hsih in order to obtain DNA for subsequent amplification (Office Action; pg. 6; citing MPEP 2142). The Patent Office further cites a KSR decision to conclude that a person of ordinary skill in the art "would have recognized the appropriate enzyme combinations for the specific cellular types." (Office Action; pg. 7). Applicants respectfully disagree with this conclusion. First, the same court also noted that the analysis supporting a rejection under 35 U.S.C. 103 should be made explicit. *KSR International Co. v. Teleflex Inc.*, 550 U.S. 398 (2007). In other words, "rejections on obviousness cannot be sustained with mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006). (MPEP 2142). Simply stating that it would have been obvious to a person of ordinary skill in the art to add known lysis agents for known bacteria to the methods of Hsih in order to obtain DNA for subsequent amplification is insufficient without articulating why it would have been obvious to do so. More specifically, the Patent Office failed to provide any rational underpinning to support its contention that a skilled artisan would use the lytic enzyme, a nonionic surfactant and a protein denaturant in a method of Hsih. Just because these components were known, does not mean their combination is obvious. Since "impermissible hindsight must be avoided and the legal conclusion must be reached on the basis of the facts gleaned from the prior art" (MPEP 2142), the Patent Office failed to show any support for combining the disclosures of Kearney and Brasher with the method of Hsih.

Moreover, applicants respectfully assert that the present invention cannot be readily conceived by combining the cited references because the claimed invention exerts a

significant and particular effect that cannot be gleaned from the teachings the cited referenced alone or in combination. For instance, Hsih describes a method of detecting *Salmonella typhimurim* and *Listeria monocytogenes* in a food sample, by combining IMS (immunomagnetic separation) and multiplex PCR. Hence, at least two different steps (IMS and multiplex PCR) are necessary. Since, it is difficult to actually perform detection with only IMS, it was a common practice at the time of filing of the present invention to subsequently perform a PCR or ELISA for actual detection.

Furthermore, in IMS, a step of pre-enriching a sample is usually necessary (see Exhibit A and Exhibit B). In fact, since the level must be equal or more than 1000 cells/mL in a sample, the bacteria that were not recognized by IMS will not be detected by performing a multiplex PCR. It was recognized by those skilled in the art that the accuracy based on such an approach would be low in an actual tests using foods. For example, applicants have tested and confirmed that "all of the bacteria could be detected with any of the methods, as long as it exists in an amount of 1 CFU/25 g." (pg. 33 of the specification as filed). However, harmful pathogenic bacteria such as pathogenic *Escherichia coli* O157, *Salmonella spp.* and *Listeria monocytogenes*, would normally produce a "negative" result in foods, i.e., allegedly not present. Therefore, a method with superior accuracy compared to that of official methods was required for the detection. The present multiple detection method filled that void and can detect with superior accuracy plural bacteria contained in foods, for example, without even using IMS.

Therefore, the contention proposed by the Patent Office that "a person of ordinary skill in the art at the time of invention would have recognized the appropriate enzyme combinations for the specific cellular types, thereby coming to the combination recited in the claimed combination" might seem conceptually easy in hindsight, however, the actual attainment

of the claimed method would require a great deal of undue experimentation based on the facts gleaned from the cited art (MPEP 2142) in order to test various possible combinations to obtain the superior accuracy provided by the claimed method.

In view of the aforementioned remarks and claim amendments, applicants respectfully asset that the instant invention is not made obvious by Hsih in view of Kearney and Brasher. Therefore, applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §103(a) rejection of claims 1, 2, 9, 11, 12 and 18 as being obvious over Hsih in view of Kearney and Brasher.

Claim 4 has been rejected under 35 U.S.C. §103(a) as being unpatentable over Hsih et al. in view of Kearney et al. and Brasher et al. as applied to claim 1 and in further view of Rimick et al. (U.S. 6,468,743), Buck et al. (Biotechniques, 27(3), 528-536, 1999) and Lowe et al. (Nucleic Acid Research 18(7), 1990, 1757-1761). Applicants, however, respectfully disagree with the attained conclusion.

As a response to applicants' previous arguments, the Patent Office states that applicant's arguments were not persuasive because "structural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds." (Office Action; pgs. 12-13). For support, the Patent Office mentions that homologues would be foreseeable by those of ordinary skill in the art. First, as applicants already mentioned before, the rejection must provide "some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." ***In re Kahn***, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006). (MPEP 2142). Simply stating that the compounds could be modified is not enough, which is more applicable to a composition claims. However, in the claimed method,

the sequence plays a critical role in the feasibility and accuracy of the claimed detection method of multiple microorganism. Simply because Rimick discloses a sequence would not suggest to those skilled in the art to use this sequence or a portion thereof in the claimed method. Based on the reasoning of the Patent Office, if the whole sequence, for example, of *Listeria monocytogenes* organism is known, then a specific method using an unknown primer for *Listeria moncytogenes* is *prima facie* obvious. This reasoning is incorrect. Those skilled in the art would not and could not specifically select the primer sequences of SEQ ID Nos. 5 and 6 of the present invention for the high sensitivity multi-organism detection method without undue experimentation. Essentially, as applicants already mentioned in the previous response, the disclosure of Rimick would simply suggest to those skilled in art to go on a fishing expedition to identify an appropriate primer pair from potentially million possible primer pairs. Hence, the combined teachings of Hsih, Kearney, Brasher, Rimick, Buck, and Lowe cannot be used as the basis for *prima facie* obviousness rejection. Therefore, applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §103(a) rejection of claim 4 as being obvious over the cited art.

Claim 14 has been rejected under 35 U.S.C. §103(a) as being unpatentable over Hsih et al. in view of Kearney et al. and Brasher et al. as applied to claim 1 and in further view of Bussey et al. (U.S. 6,011,148). Specifically, the Patent Office holds that "it would have been *prima facie* obvious to a person of ordinary skill in the art at the time of invention to utilize Tween 20 in the lysis mixture of Hsih since the prior art highlights Tween 20 as a functional equivalent of SDS." Furthermore, the Patent Office states that "[a]s recited above, the examiner is not proposing a single combination of all teachings from each reference without any regard for

the potential pitfalls of such combinations. (Office Action; pg. 14). Applicants respectfully disagree.

As applicants previously states regarding the method of Bussey, the chromosomal DNA is precipitated together with protein and cell debris and subsequently removed. Therefore, it cannot be conceived from Bussey disclosure to use Tween 20 in the method of the present invention where the chromosomal DNA is amplified by PCR, followed by analysis, to detect microorganisms. Therefore, to use Tween 20 in Bussey would rather teach away the use of Tween 20 in the present invention in which the amplification target is chromosomal DNA. (see MPEP 2141.02).

Therefore, the reasoning proposed by the Patent Office that "the examiner is not proposing a single combination of all teachings from each reference without any regard for the potential pitfalls of such combinations" disregards the fact that the cited reference, i.e., Bussey, in fact, teaches away. Therefore, applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §103(a) rejection of claim 14 as being obvious over the above-cited art .

Claim 15 has been rejected under 35 U.S.C. §103(a) as being unpatentable over Hsih et al. in view of Kearney et al. and Brasher et al. as applied to claim 1 and in further view of Aznar et al. (of record). For instance, the Patent Office states that "it would have been prima facie obvious to a person skill in the art at the time of invention to utilize Enterolysin A in the lysis mixture of Hsih since the prior art highlights such protein as lysis agent." Applicants respectfully disagree.

As it is stated in the above, to arrive at the present invention, there are plural reagents that can be used in a step for extracting various DNA other than the cited references. It

is an ex-post determination to say that the combination could have been readily conceived because the combination obtained as a result of trial and error partially overlaps with the above references. Such determination constitutes an impermissible hindsight and cannot serve as the basis of *prima facie* obviousness.

In view of the aforementioned remarks and claim amendments, applicants respectfully asset that the instant invention is not made obvious by Hsih in view of Kearney and Brasher and in further view of Aznar. Therefore, applicants respectfully request reconsideration and withdrawal of the 35 U.S.C. §103(a) rejection of claim 15 as being obvious over the above-cited art.

Dependent Claims

The applicants have not independently addressed all of the rejections of the dependent claims. The applicants submit that for at least similar reasons as to why independent claim(s) 1 from which all of the dependent claims 2, 4, 9, 11-15 and 18 depend are believed allowable as discussed *supra*, the dependent claims are also allowable. The applicants however, reserve the right to address any individual rejections of the dependent claims and present independent bases for allowance for the dependent claims should such be necessary or appropriate.

CONCLUSION

Based on the foregoing remarks, Applicants respectfully request reconsideration and withdrawal of the restriction requirement imposed on the pending claims and allowance of this application. Favorable action by the Examiner is earnestly solicited.

AUTHORIZATION

The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment to Deposit Account No. **50-4827**, Order No. 1004451.001US.

In the event that an extension of time is required, or which may be required in addition to that requested in a petition for an extension of time, the Commissioner is requested to grant a petition for that extension of time which is required to make this response timely and is hereby authorized to charge any fee for such an extension of time or credit any overpayment for an extension of time to Deposit Account No. **50-4827**, Order No. 1004451.001US.

Respectfully submitted,
Locke Lord Bissell & Liddell LLP

Dated: February 10, 2011

By: /Serge Ilin-Schneider/
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Exhibit A

invitrogen

Cat. no. 710.06

Rev. no. 004

Dynabeads® anti-Listeria

For rapid selective isolation and concentration of Listeria

For laboratory use only.

Not for use in human diagnostic procedures.

PRODUCT DESCRIPTION

Dynabeads® anti-Listeria is made of uniform, paramagnetic, polystyrene beads and purified anti-Listeria antibodies, which are bound covalently onto the surface. The antibody coated beads are supplied in a suspension of phosphate buffered saline (PBS) pH 7.4 with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3).

PRINCIPLE

Dynabeads anti-Listeria is designed for a rapid isolation and concentration of *Listeria* directly from pre-enriched samples using immunomagnetic separation (IMS). An aliquot of the pre-enriched sample is incubated with Dynabeads anti-Listeria and the antibodies coated onto Dynabeads will specifically bind *Listeria* and form a complex. The Dynabeads-Listeria complexes are subsequently separated and isolated from the sample matrix using a magnetic particle concentrator, Dynal® MPC™-S.

INTENDED USER

Any laboratory skilled in using conventional microbiological techniques, equipped and/or certified to do *Listeria* testing on food, feed and environmental samples may use Dynabeads anti-Listeria. The user must be skilled in using conventional microbiological techniques and interpreting results.

SAMPLE MATRIX

IMS with Dynabeads anti-Listeria can be done on any food, feed or environmental sample that has been pre-enriched for 24 hours in Half Fraser broth. Environmental samples include water filtrate, surface swab streaks or faecal swabs of animal origin. Food is defined as material used for human consumption and feed is defined as material used for animal consumption.

ANALYTE

Dynabeads anti-Listeria reacts against all *Listeria* monocytogenes serotypes but shows a reduced reaction to all other *Listeria* species.

INTERPRETATION CRITERIA

The test is based on either plating the concentrated Dynabeads-Listeria complexes onto internationally accepted *Listeria* selective media, for example Palcam and modified Oxford agars. Chromogenic *Listeria* plating media may also be used to supplement colony identification. Interpretation of presumptive results depends on the skill of the user to correctly differentiate the isolated colonies based on the typical *Listeria* morphology. Suspect colonies must be confirmed using standard biochemical and serological test methods.

Factors that affect the products performance

The performance of Dynabeads anti-Listeria is dependent on the extent of particle recovery from different sample matrices. Failure to recover the Dynabeads-Listeria complexes could result in failure to detect the presence of *Listeria* in an otherwise positive sample. In extremely fatty, viscous and/or particulate samples a two-fold dilution of the 24 hours enriched sample with the wash buffer must be made prior to IMS analysis. Such a dilution will not limit detection of *Listeria* but rather ensure that Dynabeads are recovered. The user must practice care not to aspirate and discard the isolated Dynabeads-Listeria complexes. To prevent loss of these complexes, leave approximately 100 µl of the original sample in the tube and dilute further by adding 1 ml of wash buffer (step 6-8, protocol B). Follow the remaining processing steps as described. The entire IMS procedure (see protocol B) shall be performed on a bench top at room temperature ranging from 18-28°C. Alternatively, automated IMS could be performed using the BeadRetriever™, in which case all performance parameters have been fully optimised and therefore are not dependent on operator aptitude.

INSTRUCTIONS FOR USE

The following protocol applies to all samples. All of the discarded material should be placed in appropriate microbiological containers and autoclaved.

A. Sample preparation

Food samples

1. Weigh 25 grams of sample material and place into a stomacher-bag with filter and add 225 ml of Half Fraser broth. A stomacher-bag with filter removes particulate material and fatty substances, which are inhibitory to IMS. (For certain foods, for example meat with bones or dry pasta, a blender is preferred prior to using a stomacher bag to avoid the risk of perforation. After blending, the contents should be transferred into a stomacher bag with a filter).
2. Incubate the prepared sample in the stomacher bag for 24 hours at 30°C
3. Mix the stomacher bag pre-enriched samples thoroughly by homogenising once more. Pipette 1 ml aliquot from the filtered section for the IMS procedure in Section B.

Environmental samples

1. Take a swab sample from any surface material or filter 10 litres of water through a membrane filter.
2. Place the swab or filter into an appropriate container filled with 10 -50 ml of pre-enrichment broth. Incubate for 24 hours at 30°C.
3. Mix by shaking vigorously and pipette 1 ml aliquot for the IMS procedure in section B.

B. Immunomagnetic separation (IMS)

1. Remove the magnetic plate and load the necessary number of 1.5 ml microcentrifuge tubes into the Dynal MPC-S.
2. Resuspend Dynabeads anti-Listeria until the pellet in the bottom of the vial disappears by using a vortex machine. Pipette and dispense 20 (l into each microcentrifuge tube.
3. Add the 1 ml from the pre-enriched sample aliquot in section A, step 3 and close the tube. Change to a new pipette for each new sample.
4. Invert the Dynal MPC-S rack five times. Incubate at room temperature for 10 minutes with gentle continuous agitation to prevent the beads from settling (e.g. in a Dynal MX sample mixer).
5. Insert the magnetic plate into the Dynal MPC-S. Invert the rack several times in order to concentrate the beads into a pellet on the side of the tube. Allow three minutes for proper recovery of beads.
6. Open the tube's cap using the tube opener provided and carefully aspirate and discard the supernatant as well as the remaining liquid in the tube's cap. (Refer to factors that affect the performance of the product). Change to a new pipette for each new sample.
7. Remove the magnetic plate from the Dynal MPC-S.
8. Add 1 ml of wash buffer (PBS-Tween). Do not touch the tube with the pipette since this can cross-contaminate the samples as well as the wash buffer. Close the tube's cap. Incubate at room temperature with gentle continuous agitation for another 10 min.
9. Repeat steps 5 - 7.
10. Resuspend the Dynabeads-Listeria complexes in 100 µl of wash buffer (PBS-Tween) and mix vigorously using a vortex mixer.

C. Isolation procedure

The resuspended Dynabeads-Listeria complexes are now ready for plating. Transfer 50 µl onto two *Listeria* plating media and plate by standard streaking with a loop or the swab-streak technique. All inoculated plating media must be incubated at 37°C. The plates are read after 24 hours and if necessary after 48 hours for presumptive *Listeria* colonies. Total analysis time from sample receipt to presumptive results is 48 hours.

D. Confirmation

The presumptive *Listeria* colonies must be confirmed by standard biochemical and serological testing or by genetic fingerprinting to identify the species.

False negative/positive results

Dynabeads anti-Listeria may record false negative results if bead recovery was particularly low and/or the level of *Listeria* species present were below 1,000 cells/ml of enriched sample. Following good laboratory practices, false positive results do not occur since the possibility to verify presumptive colonies is always applicable.

Specificity and sensitivity

The recommended protocols for use with Dynabeads anti-Listeria will determine the presence or absence of one viable *Listeria* in 25 grams of sample If this one cell is able to replicate and nor competed out by resident background flora during the 24 hours enrichment. Dynabeads anti-Listeria enables visible growth of *Listeria* on a plating medium from an enriched sample containing as low as 100 *Listeria*/ml against a background of competing flora greater or equal to 106 organisms/ml. Dynabeads anti-Listeria significantly concentrates *Listeria* from a mixed culture. For example, an initial ratio of *Listeria*

versus competing flora of 1:20 is often reduced to between 1:1 to 1:2 giving a positive concentration factor ranging between 10 to 20 times. A certain degree of cross reactivity and non-specific binding is evident, but it does not affect the overall ability of the product to bind Listeria in a mixed culture.

Accuracy and precision

The accuracy of the method is not measurable since IMS is a qualitative method. More than one *Listeria* may be bound to one or more beads and form aggregates. These Dynabeads-Listeria aggregates may give rise to only one colony-forming unit on the selective plating media. It is therefore important to vortex vigorously to break up aggregates prior to plating. Precision of the method is dependent on the extent to which particles are recovered from different sample matrices.

MATERIALS NOT PROVIDED

- Micro-pipette (10-100 µl)
- 1 ml dispenser-pipette
- Half Fraser broth; commercially available from all major media manufacturers
- Stomacher and Stomacher bag with filter
- Test tubes, glass-ware, loops, swabs and pipettes
- Wash buffer (PBS-Tween): 0.15 M NaCl, 0.01 M Sodium Phosphate buffer, pH 7.4 with 0.05% Tween-20. (Autoclave the buffer at 121°C for 15 minutes). Prepared buffer can be stored under refrigeration
- Selective culture media

All reagents should be of analytical grade.

GENERAL INFORMATION

Invitrogen Dynal AS complies with the Quality System Standards ISO 9001: 2000 and ISO 13485:2003.

STORAGE/STABILITY

Dynabeads anti-Listeria is stable, when stored unopened at 2-8°C, until the expiration date stated on the label.

PRECAUTIONS/LIMITATIONS

In order to obtain a homogeneous dispersion of beads in suspension, resuspend Dynabeads anti-Listeria by using a vortex until pellet in the bottom disappears before use.

Precautions should be taken to prevent bacterial contamination of opened vials. All material that is used and contaminated should be autoclaved and properly disposed of according to local regulations.

Avoid pipetting by mouth. This product contains 0.02% sodium azide as a preservative. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. When disposing through plumbing drains, flush with large volumes of water to prevent azide build-up.

The product is not for use in human diagnostic or therapeutic procedures.

Intellectual Property Disclaimer

Invitrogen Dynal will not be responsible for violations or patent infringements that may occur with the use of our products.

Limited Use Label License

No. 5: Invitrogen Technology – The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of patents owned or controlled by Invitrogen Corporation which cover this product based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that neither this product nor any of its components was used in the manufacture of such product. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact

Licensing Department,
Invitrogen Corporation,
1600 Faraday Avenue, Carlsbad,
California 92008.
Phone (760) 603-7200.
Fax (760) 602-6500.
Email: outlicensing@invitrogen.com

WARRANTY

The products are warranted to the original purchaser only to conform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Invitrogen Dynal's obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Invitrogen Dynal's expense, of any products which shall be defective in manufacture, and which shall be returned to Invitrogen Dynal, transportation prepaid, or at Invitrogen Dynal's option, refund of the purchase price.

Claims for merchandise damaged in transit must be submitted to the carrier.

This warranty shall not apply to any products which shall have been altered outside Invitrogen Dynal, nor shall it apply to any products which have been subjected to misuse or mishandling. ALL OTHER WARRANTIES, EXPRESSED, IMPLIED OR STATUTORY, ARE HEREBY SPECIFICALLY EXCLUDED, INCLUDING BUT NOT LIMITED TO WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. Invitrogen Dynal's maximum liability is limited in all events to the price of the products sold by Invitrogen Dynal. IN NO EVENT SHALL INVITROGEN DYNAL BE LIABLE FOR ANY SPECIAL, INCIDENTAL OR CONSEQUENTIAL DAMAGES. Some states do not allow limits on warranties, or on remedies for breach in certain transactions. In such states, the limits set forth above may not apply.

PATENTS / TRADEMARKS

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Contact details for your local Invitrogen sales office/technical support can be found at http://www.invitrogen.com/contact

SPEC-08226

Exhibit B

invitrogen

Cat. no. 710.02

Rev. no. 008

Dynabeads® anti-Salmonella

For rapid, selective enrichment of Salmonella

For research use only

INDEX

1 PRODUCT DESCRIPTION

1.1 Intended Use

Dynabeads anti-Salmonella is designed for rapid, selective concentration of Salmonella directly from pre-enriched samples. This process can be automated using a BeadRetriever™ bench top instrument or performed using a manual method.

1.2 Intended User

Any laboratory skilled in using conventional microbiological technique equipped and/or certified to do Salmonella testing on food, feed and environmental samples may use Dynabeads anti-Salmonella.

The user must be skilled in using conventional microbiological techniques and in interpreting results.

1.3 Sample Matrix

Any food, water, feed or environmental sample that has been pre-enriched for 18-24 hours in a standard Salmonella pre-enrichment broth is suitable for IMS with Dynabeads anti-Salmonella.

1.4 Principle

Dynabeads anti-Salmonella is designed for rapid, selective concentration of Salmonella directly from pre-enriched samples using manual IMS or automated IMS on the Dynal BeadRetriever™.

Dynabeads anti-Salmonella may either replace or supplement the use of a selective enrichment broth stage for the isolation of Salmonella. Dynabeads anti-Salmonella are simply incubated with an aliquot of the pre-enriched sample and the antibodies coated onto the beads will specifically bind the target bacteria. The bead-bacteria complexes are subsequently separated by using a magnetic particle concentrator, Dynal MPC®-S. For automated IMS, the Dynabeads anti-Salmonella, wash buffers and samples are loaded into the BeadRetriever and all incubations and wash steps are carried out automatically in the instrument.

After IMS, Dynabeads anti-Salmonella can be used with any standard Salmonella selective plating medium to accommodate the different Salmonella testing regimes used from country to country.

Concentrated bead-bacteria complexes can be processed using either a RAPID method or an ENHANCED method.

The RAPID method is recommended for processed samples containing low resident flora. Presumptive identification is achieved 24 h sooner than with the ENHANCED method. Following the IMS process the bead-bacteria complexes are plated directly onto internationally accepted Salmonella selective media, such as Brilliant Green agar (BGA), Xylose-Lysine-Desoxycholate agar (XLD), Bismuth Sulphite agar (BSA), Hektoen agar (HE), etc.

The ENHANCED method improves the isolation of Salmonella from samples containing high resident flora. The method consists of transferring the bead-bacteria complexes into standard selective enrichment broth and then plating onto any of the above media or other chromogenic Salmonella plating media (e.g. Rambach agar) using the standard plating technique

The improved sensitivity of the ENHANCED method is due to the specific concentration of Salmonella in the pre-enriched sample during IMS and the significant lowering of the initial ratio between Salmonella species (spp.) and background flora. The subsequent transfer of the bead-bacteria complex into Rappaport Vassiliadis Soya peptone broth (RVS) gives the Salmonella spp. a growth advantage due to the further inhibition of the competitive flora.

The ENHANCED method can be used for all food categories, except shell eggs (see section 2.1). Refer to the national reference method for analysing Salmonella (i.e. BAM, ISO etc.) when a particular food material (e.g. cocoa powder, spices etc.) requires special sample treatment and incubation media. The special sample treatment will not interfere with IMS but will only enhance the detection of Salmonella in these particular samples.

For both methods the recommended swab-streak technique should be used when plating the bead-bacteria complexes as this will result in improved colony isolation on culture media.

1.5 Interpretation Criteria

Interpretation of presumptive results depends on the skill of the user to correctly differentiate the isolated colonies based on typical Salmonella morphology. Suspect colonies must be confirmed by standard biochemical and serological test methods.

1.6 Description of Materials

Dynabeads anti-Salmonella are uniform, superparamagnetic, polystyrene microscopic beads with affinity purified antibodies against Salmonella covalently bound to the surface. The beads are supplied in a suspension of phosphate buffered saline (PBS) pH 7.4 with 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3).

Sufficient Dynabeads anti-Salmonella are provided to perform 250 tests (Product Number 710.02).

Additional Materials & Equipment Required & Supplied By Invitrogen Dynal

For performing AIMS:

Component	Prod No.	Pack Size
BeadRetriever instrument	159.50	1 Unit
A bench top instrument for performing automated IMS		
BeadRetriever Tubes and Tips	159.51	240 Tests
Disposable sample tube strips and lip combs to protect the magnetic probes for use in the BeadRetriever		

For performing manual IMS:

Component	Prod No.	Pack Size
Dynal Magnetic particle concentrator®(MPC)-S	120.20D	1 Unit
Dynal-MIX	150.10	1 Unit
Dynal Sample Mixer (US only)	947.01	1 Unit

All reagents should be of analytical grade.

Additional Materials & Equipment Needed & Not Supplied By Invitrogen Dynal

- Micropipette (10 - 100 µl)
- 1 ml dispenser Pipette
- Stomacher apparatus and stomacher bag with filter
- Test tubes, glasswork, loops, swabs
- Washing buffer (PBS Tween): 0.15M NaCl, 0.01M Sodium-Phosphate buffer, pH 7.4, with 0.05 % Tween-20. (Autoclavable at 121°C for 15 minutes)
- Pre-enrichment broths such as buffered peptone water (BPW)
- Enrichment and selective culture media

All reagents should be of analytical grade.

2 PROTOCOLS

2.1 Sample Preparation

Weigh 25 g of sample material and place into a stomacher bag with filter and add 225 ml of pre-enrichment broth. (Invitrogen Dynal recommends buffered peptone water as a pre-enrichment broth)

Mix well using the stomacher apparatus (A stomacher-bag with filter removes particulate matter as well as fatty components and allows easy pipetting of clear aliquot for analysis). For certain foods, for example bony meat, pasta, etc. a blender is preferred prior to using a stomacher bag to avoid the risk of perforation. However, after blending the contents should be transferred into a stomacher bag with a filter.

For environmental samples using a swab, place the swab into 10-50 ml of pre-enrichment broth and incubate as described below.

Incubate the prepared sample in the stomacher bag for 16-24 hours at 37°C. Mix the pre-enriched sample thoroughly by homogenising once more. Pipette 1 ml aliquot of the filtered suspension for the immunomagnetic separation procedure. Change to a new pipette for each new sample.

Method For Shell Eggs

Wash dirty eggs with a stiff brush under running water, and dry with a paper towel. Dip the eggs into 70% ethanol for 5-10 seconds and allow to dry. Alternatively follow any standard procedure for disinfecting shell eggs.

Aseptically crack open the eggs and mix/blend thoroughly both whites and yolk. Add Ferrous Sulphate ($FeSO_4$) solution to a final concentration of 35 mg/L. Pre-incubate the egg mixture at 37°C for 6 hours.

After pre-incubation, mix the egg mixture thoroughly. Dilute an aliquot five fold with wash buffer or buffered peptone water and use 1 ml of this dilution for IMS analysis. Use a new pipette or a new pipette tip for each sample to avoid cross-contamination. Re-incubate the remaining undiluted egg mixture overnight at 37°C.

2.2 Performing The Immunomagnetic Separation

2.2.1 Automated Immunomagnetic Separation (Aims) Using Dynabeads Anti-Salmonella & BeadRetriever

1. Load one BeadRetriever sample tube strip for each sample into a sample rack.
2. Resuspend Dynabeads anti-Salmonella by vortexing until the pellet in the bottom of the tube disappears and aseptically add 10 µl of properly mixed Dynabeads anti-Salmonella into sample tubes 1 and 2.
3. Aseptically add 500 µl of wash buffer to sample tubes 1 and 2.
4. Aseptically add 1 ml of wash buffer to tubes 3 and 4 within the strip.
5. Aseptically add 100 µl of wash buffer to tube 5.
6. For each sample remove the labelled sample tube strip from the sample rack and place in a second sample rack (one metre away). Add 500 µl of the test sample to tubes 1 and 2 and return the inoculated tube to the first sample rack. Repeat for the remaining samples.
7. Aseptically insert the sterile protective sample tip combs into the instrument.
8. Insert the rack with filled tubes into the instrument to lock it in place.
9. Check that everything is properly aligned and close the instrument door.
10. Select the SALMONELLA program sequence by scrolling with the arrow key and press the START button.

NOTE: For the Shell Eggs method select the Salmonella (eggs) program from the BeadRetriever menu.

11. While the instrument is in operation, the door must be kept closed. Each processing step and the total time remaining can be followed on the LC display.
12. At the end of the program run, remove the sample rack from the instrument and, for each sample, process the bead-bacteria complexes either using the RAPID or ENHANCED methods described below.
13. Remove the sample tip combs and discard into a biohazard waste container together with the tube strips.

2.2.2 Immunomagnetic Separation – Manual IMS

NOTE: To avoid cross-contamination and for safety reasons, it is strongly recommended that IMS should be performed using the BeadRetriever. In the absence of the BeadRetriever, strict adherence to good laboratory practice and the following instructions are a prerequisite to obtaining valid results.

1. Remove the magnetic plate and load one 1.5 ml Eppendorf tube for each sample into the Dynal MPC-S.
2. Resuspend Dynabeads anti-Salmonella by vortexing until the pellet in the bottom of the tube disappears and aseptically add and dispense 10 µl into each tube.
3. Add 1 ml of the pre-enriched filtered sample aliquot and close the tube. Change to a new pipette for each new sample.
4. Invert the Dynal MPC-S rack five times to mix sample and beads. Incubate at room temperature for 10 minutes with gentle continuous agitation to prevent the beads from settling e.g. in a Dynal MX4 sample mixer).
5. Insert the magnetic plate into the Dynal MPC-S. Allow 3 minutes for proper recovery of beads. During this period, invert the rack several times in order to concentrate the beads into a pellet on the side of the tube.

NOTE: The magnetic plate for the Dynal MPC-S has two positions for insertion. The vertical position is intended for use with round-bottomed or conical microcentrifuge tubes for larger volume applications (0.5 – 2 ml). The tilted position is for conical microcentrifuge tubes only and is better for lower volume applications (0.01 - 0.5 ml).

6. Open the tube cap using the tube opener provided and carefully aspirate and discard the supernatant as well as the remaining liquid in the tube's cap taking care not to disturb the pellet of IMS beads on the side wall of the tube. Change to a new pipette for each new sample.
7. Remove the magnetic plate from the Dynal MPC-S.
8. Add 1 ml of wash buffer. Change to a new pipette for each new sample. Do not touch the tube with the pipette since this can cross-contaminate the samples as well as the wash buffer. Close the cap. Invert the rack several times to resuspend the beads.
9. Repeat steps 5-8.
10. Repeat steps 5-7.
11. Resuspend the Dynabead-bacteria complex in 100 µl of wash buffer. Mix briefly using a vortex mixer
12. For each sample process the bead-bacteria complexes using either the RAPID or ENHANCED methods described below.

2.2.3 Post IMS

The ENHANCED Method

This is the recommended method for all food and environmental samples. Presumptive Salmonella positive results are available three days after receipt of samples.

Transfer the concentrated, resuspended beadbacteria complexes into 10 ml of Rappaport-Vassiliadis Soya peptone broth (RVS) and incubate at 42°C for 18-24 hours.

Follow standard procedure for isolation by spreading a loopful of RVS culture onto any Salmonella plating media.

The RAPID Method

This is recommended for processed or foods known to harbour none or low levels of background flora only. Presumptive Salmonella positive results are available two days after receipt of samples.

Transfer 50 µl of the resuspended bead-bacteria complex onto each of two Salmonella selective agar plates. (BGA, XLD, BSA, HE etc.)

2.2.4 Dynabeads anti-Salmonella IMS For Shell Eggs

Both the Automated and Manual IMS methods are suitable for analysis of enrichment cultured from samples of raw Shell Egg.

For the automated method, follow the instructions in 2.1.1 step 1 and step 2 - resuspend Dynabeads anti-Salmonella by vortexing until the pellet in the bottom of the tube disappears and aseptically add 20 µl of properly mixed Dynabeads anti-Salmonella into sample tubes 1 and 2.

For the manual method, follow the instructions in 2.2.2 and at step 2 - resuspend Dynabeads anti-Salmonella by vortexing until the pellet in the bottom of the tube disappears and aseptically add 40 µl of properly mixed Dynabeads anti-Salmonella into each eppendorf sample tube. Proceed as directed in each method. Steps 1-11 should be repeated on a five-fold dilution of the overnight incubated samples that returned presumptive Salmonella negative results after 6 h IMS analysis.

2.3 Specificity And Sensitivity

Dynabeads anti-Salmonella reacts with all current Salmonella serovars of importance as the cause of human and animal disease occurring in food, feed and environmental samples. This currently covers somatic groups from B-Z with variable reactivity depending on the serotype.

These protocols for using Dynabeads anti-Salmonella will determine the presence or absence of one viable Salmonella in 25 g of sample if this one cell is able to replicate and not out-competed by resident background flora during the overnight preenrichment. Using Dynabeads anti-Salmonella enables visible growth of Salmonella on a plating medium from a pre-enriched sample containing as low as 100 Salmonella/ml against a background of enteric competing flora greater or equal to 10^6 organisms/ml. Dynabeads anti-Salmonella significantly concentrates Salmonella from a mixed culture. For example, an initial ratio of Salmonella versus enteric competing flora of 1:20 is often reduced to between 1:1 to 1:2 giving a positive concentration factor ranging between 10 to 20 times. A certain degree of cross reactivity and non-specific binding is evident but it does not affect the overall ability of the product to bind Salmonella in a mixed culture.

CONFIRMATION

Presumptive Salmonella colonies must be confirmed by standard biochemical and serological testing.

The accuracy of this method is not measurable since it is a qualitative, not a quantitative method. Several target bacteria may be bound to the beads and give rise to only one colony forming unit on the selective plating media. The precision is dependent on the extent to which particles are recovered from different sample matrices.

FALSE NEGATIVE/POSITIVE RATES

In seeded samples, Dynabeads anti-Salmonella records a false negative rate ranging between 5-15% depending on the inoculum, type, serovariant, matrix flora flora and sample matrix. In the same sample the conventional method ISO 6579 records a false negative rate of 5-25%. In naturally contaminated samples, Dynabeads anti-Salmonella will give a false negative rate ranging between 2.5-10%. In the same sample the conventional method ISO 6579 will give a false negative rate of 22.5-30%. Dynabeads anti-Salmonella documents the false negative rate compared to the conventional method ISO 6579. False positive rates do not occur since the possibility to verify presumptive colonies is always applicable. However the method depends on the user following good laboratory practices and avoiding cross-contamination of samples.

2.4 Factors Affecting Product Performance

- The IMS procedure should be performed on a bench-top at room temperature between 15-25°C and all reagents must be at room temperature before use.
- Ensure that the Dynabeads anti-Salmonella are fully dispersed by vigorous vortexing for at least 10 seconds before use.
- It is important that filtered pipette tips are used to transfer samples into the test tubes for both manual and automated IMS.
- In extremely fatty, viscous and/or particulate samples, a two to ten-fold dilution of the 24 h pre-enriched sample using the described wash buffer could be made prior to IMS analysis. Such a dilution will not limit detection of Salmonella but rather ensure that maximum beads are recovered.
- During bead-bacteria complex magnetic capture it is essential that gentle rocking of the Dynal MPC is continued. This prevents binding of low mass debris, which is magnetic or magnetisable
- For manual IMS the performance is solely dependent on the extent to which particles are recovered from different sample matrices.
- For manual IMS the user must practice care not to aspirate and discard the isolated bead-bacteria complexes. The use of vacuum aspirators has been shown to reduce the recovery of bacteria. Failure to recover the bead-bacteria complexes could result in failure to detect the presence of Salmonella in an otherwise positive sample.
- For automated IMS, to avoid cross-contamination of the prepared tubes, it is recommended that sample transfer into the tubes is performed in a designated area at least one metre from the prepared tubes. Sample tube-strips for the BeadRetriever are designed to fit into the rack in one direction only. Tip combs and tube tray should be inserted as instructed until a click sound is heard. At the end of the processing of a sample, remove the sample tray first before removing the tip combs. It is recommended that the tip combs remain for at least 10 minutes after the assay has been completed to allow for air-drying, before removal.

2.5 Precautions/Limitations

In order to obtain a homogeneous dispersion of beads in suspension, resuspend Dynabeads anti-Salmonella by using a vortex until pellet in the bottom disappears before use. Precautions should be taken to prevent bacterial contamination of opened vials.

All material that is used and contaminated should be autoclaved and properly disposed of according to local regulations.

The product is not for use in human diagnostic or therapeutic procedures.

3 GENERAL INFORMATION

3.1 Storage/Stability

Dynabeads anti-Salmonella is stable, when stored unopened at 2-8°C, until the expiration date stated on the label.

3.2 Technical Service

Contact details for your local Invitrogen technical support can be found at http://www.invitrogen.com/contact

3.3 Warnings and Limitations

This kit is for research use only. Follow appropriate laboratory guidelines.

This product contains 0.02% sodium azide as a preservative, which is cytotoxic. Avoid pipetting by mouth!

Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. When disposing through plumbing drains, flush with large volumes of water to prevent azide build up.

Certificate of Analysis (CoA) is available upon request.

Material Safety Data Sheet (MSDS) is available at www.invitrogen.com

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3.6 Warranty

The products are warranted to the original purchaser only to perform to the quantity and contents stated on the vial and outer labels for the duration of the stated shelf life. Invitrogen Dynal's obligation and the purchaser's exclusive remedy under this warranty is limited either to replacement, at Invitrogen Dynal's expense, of any products which shall be defective in manufacture, and which shall be returned to Invitrogen Dynal, transportation prepaid, or at Invitrogen Dynal's option, refund of the purchase price.

Claims for merchandise damaged in transit must be submitted to the carrier.

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4 REFERENCES

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AOAC - Samples of this test kit model were independently evaluated by the AOAC Research Institute and were found to perform to the producer's specifications as stated in the test kit's descriptive insert. The producer certifies this kit conforms in all respects to the specifications originally evaluated by the AOAC Research Institute as detailed in the 'PERFORMANCE TESTED' certificate number 970401.

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